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| <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; padding: 5px;"> <p>(21) International Application Number: PCT/GB99/03059</p> <p>(22) International Filing Date: 14 September 1999 (14.09.99)</p> <p>(30) Priority Data: 9820014.0 14 September 1998 (14.09.98) GB</p> <p>(71) Applicant (for all designated States except US): CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED [GB/GB], Cambridge House, 6-10 Cambridge Terrace, Regent's Park, London NW1 4JL (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): GRAHAM, Gerard, John [GB/GB], Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD (GB). NIBBS, Robert, John, Benjamin [GB/GB], Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD (GB).</p> <p>(74) Agents: BRASNETT, Adrian, H. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).</p> </td> <td style="width: 50%; vertical-align: top; padding: 5px;"> <p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> </td> </tr> </table> | | | <p>(21) International Application Number: PCT/GB99/03059</p> <p>(22) International Filing Date: 14 September 1999 (14.09.99)</p> <p>(30) Priority Data: 9820014.0 14 September 1998 (14.09.98) GB</p> <p>(71) Applicant (for all designated States except US): CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED [GB/GB], Cambridge House, 6-10 Cambridge Terrace, Regent's Park, London NW1 4JL (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): GRAHAM, Gerard, John [GB/GB], Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD (GB). NIBBS, Robert, John, Benjamin [GB/GB], Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD (GB).</p> <p>(74) Agents: BRASNETT, Adrian, H. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).</p> | <p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> |
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| <p>(54) Title: MIP-1α ISOFORM LD78β AND USES THEREOF</p> <div style="text-align: center; font-family: monospace; font-size: 0.8em;"> <pre> hcrantes ----- -ePTSSPT.T PCGFAIARF LPRAMISST Y.TSERCENF AVVYTRION QVCADPEEN VREYINLEN S----- + + hmip-1β ----- -aPMSDEPT ACCFSYXAK LPRMTVDYX E.TSRLCSQP AVVYQKXKX QVCADPEEN VQKVSDELEL N----- + + nmip-1β ----- -aPMSDEPT SCGFSYXKQ LPRMTVDYX E.TSRLCSQP AVVYQKXKX QVCADPEEN VQKVSDELEL N----- + + hMIP-1α { hLD78α/MIP-1αS ----- -aSLAODEPT ACCFSYXKQ IPQNTIADT E.TSSQCCKP GVYFLXKXK QVCADPEEN VQKVSDELEL SA----- - - { hLD78β/MIP-1αP ----- -aFLAODEPT ACCFSYXKQ IPQNTIADT E.TSSQCCKP GVYFLXKXK QVCADPEEN VQKVSDELEL SA----- ? ? { nmip-1α ----- -aFLAODEPT ACCFSY.SRK IPQNTIADT E.TSRLCSQP GVYFLXKXK QVCADPEEN VQKVSDELEL NA----- + + </pre> </div> | | | | |
| <p>(57) Abstract</p> <p>The present invention relates to the finding that the isoform of the chemokine MIP-1α, referred to as LD78β or MIP-1αP, has high suppressive activity against the CCR5 co-receptor, implicated in the entry of HIV into cells. The invention provides methods to block entry of HIV into cells using MIP-1αP, the use of MIP-1αP in the treatment of HIV and a novel form of this chemokine, amino-oxypentane MIP-1αP, which has particularly advantageous properties.</p> | | | | |

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MIP-1 α ISOFORM LD78 β AND USES THEREOF

The present invention relates to an isoform of the chemokine MIP-1 α and its use as a receptor antagonist.

5

Background to the Invention.

The mammalian immune system is controlled by a complex series of interactions between signalling molecules. One group of
10 such molecules is the β -chemokines, which are a family of proteins of a molecular mass of about 8,000 kD. Chemokines play diverse roles in both inflammatory and non inflammatory situations¹ via activation of a family of heptahelical G protein coupled receptors². In addition, they have been
15 implicated in the pathogenesis of HIV (ref. 3). Chemokines are active on lymphocytes and monocytes by means of these G protein coupled receptors. Of this G-protein family, the receptor CCR5 is of particular significance in medicine. This receptor is implicated in the transmission of HIV-1
20 since this virus requires CCR5 as a co-receptor (along with CD4) for entry into T-cells. A truncated allele of CCR5 (which causes the loss of the three C-terminal transmembrane domains) results in resistance to HIV-1 infection or a reduction in the severity of disease progression (Samson et
25 al, Nature 382; 722-725, (1996)).

It has been reported that the β -chemokines RANTES, MIP-1 α and MIP-1 β are HIV-suppressive factors produced by CD8⁺ T-cells (Ref. 4). Specifically, the chemokines MIP-1 α , MIP-1 β and
30 RANTES have been shown to be major HIV1 suppressive factors produced by T cells⁴. Subsequently, the chemokine receptor, CCR5, which recognises these ligands, was shown to serve as a critical cofactor mediating the entry of M-tropic HIV1 strains⁵⁻¹¹. Current data suggests RANTES is the most

effective natural antagonist of CCR5-dependent HIV1 entry, whilst MIP-1 α is somewhat less efficient^{5,9,12}.

It is believed that the binding of these factors to the CCR5
5 receptor is responsible at least in part for the suppressive effects of these β -chemokines. Current data suggest that RANTES is the most effective natural antagonist of CCR5-dependent HIV-1 entry into CD4 cells.

10 The human MIP-1 α chemokine has been found to exist in two non-allelic isoforms (Refs 13, 14). The term MIP-1 α , when used without qualification, is generally used to refer to the more widely used isoform, also called LD78 α , the predominant experimentally used isoform. The other isoform, LD78 β ,
15 differs in its mature sequence by three amino acids (see Figure 1). Of these, one is the substitution of serine at position 2 of the LD78 α sequence by proline.

No functional significance attributed to this difference has
20 been described, and indeed the widespread experimental use of the LD78 α isoform suggests that those of skill in the art do not attach any major significance to this difference. This may be because it has been reported that the MIP-1 α expressed in some cells is a truncated version of the LD78 α sequence
25 shown herein in Figure 1, in which the first 4 residues of the sequence are missing (e.g. Cocchi et al, Ref. 4). This truncation removes this region of difference between the isoforms.

30 Disclosure of the invention.

Recently, we have cloned a chemokine receptor which we have termed D6 (refs. 15-16). Curiously, whilst murine and human D6 bind murine MIP-1 α with high affinity, the closely related
35 human MIP-1 α interacts poorly with these receptors.

Similarly, murine CCR5 binds murine MIP-1 α with high affinity but does not recognise the putative human homologue¹⁵. The isoform of human MIP-1 α that we have used in these studies is a commercially available form that has the amino acids ASLA at the amino terminus, and has previously been referred to as LD78 α to distinguish it from a naturally occurring non-allelic variant, LD78 β (refs. 13-14). The close sequence similarity between LD78 α and murine MIP-1 α led us to examine the possible amino acid residues that may be responsible for the discriminatory binding of these ligands. As shown in Figure 1, the only consistent feature present in the mCCR5 and hD6 ligands but absent in human LD78 α is the proline residue at position 2 of the mature protein. LD78 β , however, does have a proline residue in position 2, with two reciprocal serine/glycine swaps in the region between cysteines 3 and 4 being the only other differences between the mature LD78 α and β peptides (Figure 1). Also, consideration of other differences in the putative signal sequences of the two isoforms¹⁴ by predictive algorithms (not shown), suggested there may be variation in the site of signal peptidase cleavage between the two isoforms during secretion, and that LD78 α may not be produced with the ASLA amino terminal amino acids. Indeed, human MIP1 α has been shown to be produced naturally by CD8+ T cells⁴ as a -4 variant with the amino terminal sequence ADTPT. We have worked extensively with the LD78 β isoform¹⁷ and numerous amino terminal sequencing exercises have consistently revealed a 'full length' amino terminus APLAADTPT, suggesting that the -4 isoform identified in T cells is likely to be a naturally generated form of LD78 α (henceforth referred to as LD78 α -4) perhaps indicating alternative signal peptidase cleavage site usage by the two LD78 isoforms.

As a result of our observations, we have surprisingly found that LD78 β , unlike the more commonly used α isoform, has high

affinity for the promiscuous β -chemokine receptor D6, is the most potent natural CCR5 agonist and displays an HIV1 suppressive activity that is markedly higher than any other known natural CCR5 ligands. A proline residue at position 2 of LD78 β is implicated in its enhanced activity and we propose that LD78 α and β be renamed MIP-1 α S and MIP-1 α P respectively to reflect their functional differences. In addition, our studies suggest that MIP-1 α P more accurately represents the human functional homologue of murine MIP-1 α .

The present invention thus provides a method of inhibiting the entry of an HIV-1 virus into a CD4⁺ cell which method comprises:

bringing said cell and said virus into contact in the presence of a polypeptide comprising the LD78 β isoform of MIP-1 α under conditions wherein said polypeptide binds to a CCR5 receptor on the surface of said cell and inhibits entry of said virus into the cell.

The invention further comprises a diagnostic method for determining the likely effectiveness of LD78 β therapy in a patient infected with, or at risk of infection from, HIV, which method comprises:

isolating a sample containing CD4⁺ T-cells from said patient;

bringing said T-cells into contact with a polypeptide comprising the LD78 β isoform; and

determining the extent to which said polypeptide can bind to said cells.

In the foregoing method, the sample may be a human body sample, such as blood or serum.

The present invention further provides the use of the polypeptide comprising the LD78 β isoform of MIP-1 α for the

manufacture of a medicament for the treatment of HIV infection.

5 The polypeptide comprising the LD78 β isoform of MIP-1 α may be in the form of the wild-type protein or may comprise one or more side-chain, N-terminal or C-terminal modifications. For example, the polypeptide may be produced by expression by recombinant means known per se in the form of a fusion protein, wherein said LD78 β isoform is fused to a second
10 polypeptide sequence, for example a signal sequence which directs the fusion protein out of the host cell.

The LD78 β isoform may also be chemically linked to groups, such as N-terminal protecting groups. For example, Simmons
15 et al (Science 276, 276-279, 1997) describe modification of RANTES by N-terminal protection with amino-oxy pentane (AOP), Such a group, or another amino-oxy alkane (e.g. C2-10 alkane) may be linked to the N-terminal of LD78 β using analogous methodology. WO96/41813 provides methods for the
20 transamination of the N-terminal of polypeptides (citing Dixon & Fields, Meth. Enzymol. 25, 409-419, 1979) and the subsequent protection of the N-terminal of such modified polypeptides with polyethylene glycol and derivatives thereof.

25 These and other methods known in the art may be used to modify LD78 β to provide polypeptides useful in the present invention, for example in the diagnostic and other methods described herein.

30 The copy number of LD78 β varies between individuals, with up to six copies being present in some humans. The present findings indicate that the progreesion of HIV infection in an individual may be related, at least in part, to the copy
35 number of LD78 β . Thus in a further aspect, the invention

provides a method for the diagnosis of an individual's susceptibility to the development or progression of HIV infection, which method comprises measuring the copy number of LD78 β in an individual's genome, or measuring the level of expression of LD78 β in the individual. Where increased copy numbers (i.e. more than one per haploid genome) are found, then the individual may be more resistant to the effects of HIV and to the progression of the infection into AIDS. The individual may be someone with or without HIV infection, or with unknown HIV status.

Description of the Drawings.

Figure 1 shows an alignment of the predicted protein sequence of some mature β -chemokines. Human and murine sequences are prefixed with 'h' or 'm' respectively. The two human MIP-1 α isoforms (hLD78 α and β) are indicated with '{'. The proline residue at position 2 (serine in hLD78 α), and the reciprocal serine/glycine changes between LD78 α and β , are in bold and underlined. Binding to human D6 and murine CCR5 is indicated to the right of the alignment, and is derived from references 16 and 15 respectively.

Figure 2 shows the displacement of I¹²⁵-mMIP-1 α from CHO cells expressing human MIP-1 α receptors (A, Human D6; B, Human CCR5; C, Human CCR1) by human MIP-1 α / LD78 isoforms.

Figure 3 shows dose-response curves for ligand-induced calcium ion fluxes in HEK293 cells expressing human MIP-1 α receptors (A, CCR5-293 cells; B, CCR1-293 cells).

Figure 4 shows that LD78 β is a more potent inhibitor of HIV entry through CCR5 than other MIP-1 α variants and RANTES (A, macrophage tropic strain JR.FL; B, strain ADA; C & D, SF162).

Figure 5 shows that AOP-LD78 β has a higher binding affinity for CCR5 than unmodified LD78 β .

Figure 6 shows that AOP-LD78 β exerts its improved effects through down-regulation of cell surface receptors.

Detailed Description of the Invention.

Reference herein to the "LD78 β isoform" means the protein of the sequence shown herein in Figure 1, or a variant thereof which retains the CCR5 binding ability of LD78 β but which differs by from 1 to 10, such as 2, 3, 4, 5 or 6 amino acids. A difference is one substitution, deletion or insertion. Where the difference is a substitution, the substitution may be conservative or non-conservative. Conservative substitutions may be made for example according to the following table, where amino acids on the same block in the second column and preferably in the same line in the third column may be substituted for each other:

| | | |
|-----------|-------------------|---------|
| ALIPHATIC | Non-polar | G A P |
| | | I L V |
| | Polar - uncharged | C S T M |
| | | N Q |
| | Polar - charged | D E |
| | | K R |
| AROMATIC | | H F W Y |

Alternatively, any amino acid may be replaced by a small aliphatic amino acid, preferably glycine or alanine.

Where the LD78 β is a variant of the sequence shown in Figure 1, the difference will be other than at position 2, where the proline residue will be retained. Preferably, the difference is also other than in the N-terminal 12 amino acids, up to

and including the cys-cys pair, and also other than at any other cysteine residue.

5 A particularly preferred class of deletions or substitutions is the deletion or substitution of a charged amino acid, particularly one in the C-terminal region of the LD78 β . We have previously found that such changes result in a protein which does not aggregate to the same extent as the corresponding wild type protein, thus making it more
10 effective as an antagonist.

Substitutions of a charged amino acid which result in a loss of overall charge are preferred. Charged amino acids in the LD78 β sequence of Figure 1 are D6, R18, D27, E30, K37, K45,
15 R46, R48, D53, E56, E57, K61, D65 and E67. Preferably, E67 is substituted, either alone or in combination with one or more of D53, E56, E57, K61 and D65.

Particularly preferred specific substitutions may be lys (K) to asn (N), asp (D) to asn (N), or glu (E) to gln (Q).
20

Thus for example a specific LD78 β isoform which may be used in the invention is that shown in Figure 1 apart from the substitutions E67Q, or E67Q + D65N, or E65Q + D65N + E57Q.
25 These and other isoforms which disaggregate may be made by methods analogous to those described in, for example, WO94/24285.

Where a polypeptide comprising the LD78 β isoform is to be
30 used in a method of inhibiting the entry of an HIV-1 virus into a cell, the cell will be a CD4⁺ cell which is also expressing a CCR5 receptor capable of acting as a co-receptor for the HIV-1 virus.

The cell may be any mammalian cell type, which either naturally expresses both CD4 and CCR5, or which has been genetically engineered to express these receptors. Examples of the latter are illustrated in the accompanying examples, and also widely available in the art, given the widespread interest in developing AIDS therapies which target the CCR5 receptor. Suitable cells include cultures of PBMCs, COS cells, CHO cells, HEK 293 cells and Hos (human osteosarcoma) cells.

The HIV-1 virus used in the method may be any nonsyncytium-inducing strain which uses CCR5 as a co-receptor, or an enveloped pseudotyped virus which uses this receptor, such as the JR.FL virus or ADA pseudotyped virus (for both see Deng et al, Nature 381, 661-666).

In the method of the invention, the amount of the LD78 β isoform which is present may vary according to the needs of those of skill in the art. For example, the method may be practised *in vitro* for the assay of compounds capable of binding to and/or antagonising the CCR5 receptor. Generally, the concentration of LD78 β in such assays will range from 100 pM to 100 nM, preferably from 100 pM to 10 nM. This range of concentrations will be suitable for other *in vitro* or *in vivo* purposes as well.

Similarly, where the invention is in predicting the likely effectiveness of LD78 β therapy in an individual, the concentration of polypeptide may be in a similar range.

LD78 β polypeptides may be formulated into pharmaceutical compositions. The compositions comprise the polypeptide together with a pharmaceutically acceptable carrier or diluent. Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for oral,

topical, or parenteral (e.g. intramuscular or intravenous) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods
5 include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or
10 finely divided solid carriers or both, and then, if necessary, shaping the product.

For example, formulations suitable for parenteral administration include aqueous and non-aqueous sterile
15 injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or
20 other microparticulate systems which are designed to target the polypeptide to blood components or one or more organs.

Suitable liposomes include, for example, those comprising the positively charged lipid (N[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA), those comprising dioleoyl-
25 phosphatidylethanolamine (DOPE), and those comprising 3 β [N-(n',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol).

Compositions may comprise any desired amount of the LD78 β
30 polypeptide. In part this will depend upon the intended formulation and its intended use. By way of general guidance the composition may comprise from about 1% to about 99%, for example from 10% to 90% of the LD78 β polypeptide.

The composition may comprise a mixture of more than one, for example two or three, LD78 β polypeptides.

Where *in vitro* use is contemplated, this will include *ex-vivo*, e.g. in the treatment of bone marrow other tissue from a subject which may be reimplanted into the subject after treatment.

In general, the methods of treatment will involve administering to a patient in need of treatment an effective amount of an LD78 β polypeptide (or composition thereof). Suitable routes of administration of compounds of the invention include oral or parenteral, and will depend in part upon the intended use and the discretion of the physician.

Thus the present invention also provides a method of treating an individual infected with HIV with an effective amount of an LD78 β of the invention. In particular, the treatment may provide the use of an N-terminal protected LD78 β .

At present, there is no established cure for HIV infection. Thus "treatment" includes the alleviation of symptoms, including the delay in the progression of the disease, the stimulation of the immune system to resist other illnesses, or the like, rather than necessarily cure.

Treatment of HIV infection may be by administering an LD78 β by any suitable route, including for example intravenous or subcutaneous injection.

The amount of an LD78 β polypeptide administered to a patient is ultimately at the discretion of the physician, taking account of the condition of the patient and the condition to be treated. Typical amounts of polypeptide administered may be in the region of 1 μ g/kg body weight to 10 mg/kg body

weight and preferably between 1 μ g and 1mg /kg body weight.
The amount, formulation and route of administration should be
chosen to achieve an effective level of the polypeptide of
around 50pg/ml to 10 μ g/ml circulating in blood or other
5 target tissue.

The dose for administration to a patient desirably will be
selected to balance between the need for an effective amount
of the polypeptide comprising the LD78 β isoform on the one
10 hand, and on the other, the need to avoid undesirable side-
effects such as inflammation, nausea, etc. The present
findings show that the LD78 β polypeptide is advantageous over
cytokines, including RANTES, which have been proposed for
therapeutic purposes, particularly in view of its high
15 affinity for the CCR5 receptor. For example, we have found
that in the HIV-1 entry inhibition assay, performed with the
JR.FL pseudotyped virus according to the protocol of Deng et
al (Ref. 5), which is incorporated herein by reference, LD78 β
provides 100% inhibition of entry at a concentration of 10nM,
20 compared to only 50% inhibition of entry with 100nM of LD78 α .
Similar results were also achieved with the ADA pseudotyped
virus. Thus LD78 β achieves a more complete inhibition of
entry of HIV-1 into a cell via CCR5 at a 10-fold lower
concentration. Even compared to RANTES, we have found a more
25 complete inhibition of entry. Using SF162 virus we have
shown that at 10 nM RANTES achieves about 75 % inhibition of
entry using the assay of Connor et al, Virology, 206, 935-
944, 1995, which is incorporated herein by reference, whereas
LD78 β achieves effectively 100% at the same concentration.
30 Thus it is about 1 log more active.

Accordingly, the invention allows the use of an LD78 β
polypeptide for in vitro or in vivo uses to achieve a desired
effect (particularly antagonism of HIV-1 entry to a cell) at
35 a concentration of 1 log less than the concentration of

either or both of RANTES or LD78 α would be required to achieve the same effect.

Doses may be administered continuously, e.g in the form of a drip, or at discrete intervals, e.g twice daily, daily, weekly or monthly.

The LD78 β may also be administered by way of a gene construct for delivery by gene therapy. For example, naked DNA constructs are known to be taken up by cells in muscle tissue. Alternatively, gene delivery vector systems such as those based upon viral vectors, e.g. adenoviral or retroviral vectors may be used to deliver a construct comprising nucleic acid encoding LD78 β operably linked to a suitable promoter.

The invention is illustrated by the accompanying examples.

Example 1: Binding of LD78 isoforms to murine MIP-1 α receptors.

We have therefore tested the ability of the various LD78 isoforms to interact with known murine MIP-1 α receptors stably expressed on CHO cells (Table 1). In contrast to LD78 α -4, LD78 β binds with high affinity to both murine CCR5 and D6, whilst LD78 α -4 is a significantly better ligand than LD78 β for murine CCR1. To assess the importance of the proline residue at position 2 of LD78 β we have tested two other proteins. One, LD78 β -4, has the first four amino acids (APLA) of LD78 β removed, and a second, LD78 α , has the four amino acids ASLA reintroduced onto LD78 α -4. Both of these proteins behaved like LD78 α -4 on the murine receptors, though there was a slight decrease in binding to mCCR1 by LD78 α . This is shown in Table 1.

Table 1

| | LD78 β | LD78 α -4 | LD78 β -4 | LD78 α |
|--------|--------------|------------------|-----------------|---------------|
| mCCR-1 | 35 | 4 | 3 | 13* |
| mCCR-5 | 1.1 | 133 | 127 | 136* |
| mD6 | 5.5 | >200 | >200 | >200* |

Table 1: Dissociation constants (in nM) for displacement of I^{125} -mMIP-1 α from CHO cells expressing murine MIP-1 α receptors, by different forms of human MIP-1 α .

Displacement curves were generated like those shown in Figure 2.

Dissociation constants were calculated from these data using LIGAND software²⁵. * indicates data taken from reference 15.

Chemokine sources: LD78 α is 'human MIP-1 α ' purchased from Peprotech, London, UK; LD78 α -4 is 'human MIP-1 α ' purchased from R&D Systems, Abingdon, UK; LD78 β was prepared as described in reference 17 and LD78 β -4 prepared by similar methods.

Example 2: Binding of LD78 isoforms to human receptors.

CHO cells expressing human receptors were also tested and showed similar results. Experiments were performed as described in refs 15 and 16, using I^{125} -labelled murine MIP-1 α at a constant concentration of 600pM (for D6), 3nM (for CCR1) or 9nM (for CCR5), whilst increasing the concentration of unlabelled competitor LD78 protein. Remaining radioactivity bound after 90min, and three ice-cold PBS washes, was determined. Each point was done in triplicate, the average taken, and converted into a percentage of radioactivity bound in the absence of any unlabelled competitor chemokine.

Thus, with human D6, whilst the LD78 β isoform binds with high affinity (Kd 5.5nM), the -4 variants of the two isoforms bind only weakly (Kd 77nM for LD78 α -4 and Kd 124nM for LD78 β -4) and LD78 α , which has the addition of the four amino acids ASLA to LD78 α -4, does not exhibit an enhanced binding affinity (Figure 2A, \square = LD78 β ; \diamond = LD78 β -4; \circ = LD78 α ; Δ = LD78 α -4). With human CCR5, there is an ~6-fold higher binding affinity of LD78 β (Kd 6.2nM) than is seen for the -4

LD78 proteins (K_ds 35nM), and the addition of the ASLA amino acids to LD78 α -4 results in a further reduction in binding affinity (Figure 2A, symbols as Fig. 2A). These results show that the proline residue at position 2 of LD78 β is
5 responsible for the enhanced binding of this isoform to murine and human D6 and CCR5. Conversely, human CCR1 binds the -4 variants of the LD78 isoforms consistently better than the 'full-length' proteins (Figure 2C, symbols as Fig. 2A).

10 Example 3: Signalling through human CCR5 and CCR1 receptors.

These isoform binding variations are also reflected in the dose response for signalling through human CCR5 and CCR1, as assayed by Ca²⁺ flux into HEK293 cells stably transfected with
15 these genes.

Experiments were performed according to Nibbs et al¹⁵. ~6x10⁶ cells loaded with Fura-2-AM were incubated at 37°C in a continuously stirred cuvette in a Perkin-Elmer LS50
20 Spectrometer (340nm (l_{ex}); 500nm (l_{em})) and fluorescence emission recorded every 100ms. After 2min, ligand was added to a defined concentration and fluorescence recorded every 100ms for a further 2min. To allow to control for variations in Fura-2 loading and cell number, cells were lysed with
25 detergent and fluorescence emission recorded. Also, to control for day-to-day experimental variation, a full dose-response curve for murine MIP-1 α was performed each time a different ligand was tested (data not shown). The peak of ligand-induced fluorescence increase (after alterations to
30 account for these variations) is presented as a percentage of the maximum achievable peak with each particular transfected cell line.

With 'full-length' proteins on CCR5, half maximal signalling
35 potency is seen at approximately 10nM for LD78 α and at

approximately 750pM for LD78 β (Figure 3A; \square = LD78 β ; \diamond = LD78 β -4; \circ = LD78 α ; Δ = LD78 α -4; X = hRANTES; \oplus = hMIP-1 β).

LD78 α was unable to induce a Ca²⁺ flux of the same magnitude as those seen with LD78 β , even when it is added at very high concentrations (>1mM) (not shown). The LD78 α -4 variant shows a slight increase in signalling potency through CCR5 compared to LD78 α , whilst removal of the terminal 4 amino acids from the β isoform reduces its activity approximately 10-fold.

The potency of signalling with LD78 β is 5-10 times that seen with RANTES and ~100-fold that seen with MIP-1 β , and is therefore the most active CCR5 agonist described to date. We have detected Ca²⁺ fluxes with MCP2, which has recently been identified as a CCR5 ligand¹⁸, but this ligand is not as potent as MIP-1 β in this assay. Signalling through human CCR1 reflects the results from the binding studies and suggests little difference in signalling potency between the 'full-length' α and β isoforms of LD78 (Figure 3B, symbols as Fig. 3A). The -4 variants however have a slightly higher signalling potency than the full length peptides. We have so far been unable to demonstrate a signalling role for human D6 (ref. 16) and so the functional consequences of the high affinity binding of LD78 β to this receptor remain to be determined.

Example 4: LD78 β as an HIV suppressive agent.

Given the very high affinity binding of LD78 β to CCR5, we investigated the potency of this chemokine as an HIV suppressive agent. Studies were carried out to investigate the potency of 'full length' and -4 variants of both isoforms of LD78 in suppressing entry of JR.FL envelope pseudotyped virus into CEMx174-CCR5 cells.

5x10⁴ CEMx174-CCR5 cells, pretreated with chemokine for 30min, were incubated for 4 hrs with luciferase virus (5ng p24), pseudotyped by either JR.FL (Fig. 4A; □ = LD78β; ◇ = LD78β-4; ○ = LD78α; △ = LD78α-4; + = AOP-RANTES) or ADA (Fig. 4B, symbols as Fig. 4A) according to protocol previously described⁵. Medium was then changed and the luciferase activity measured 3 to 5 days post-infection. The extent of inhibition of HIV entry was determined by comparing luciferase activity of chemokine-treated cells, with untreated cells⁵.

These studies demonstrated LD78β to be a substantially more potent suppressor of HIV entry than either LD78α or the -4 variants (Figure 4A). Greater than half maximal inhibition of JR.FL pseudotyped viral entry was achieved with concentrations of LD78β as low as 5ng/ml whilst 10-20 fold higher concentrations of the other LD78 variants were required before 50% inhibition of viral entry was achieved. Similar results were seen with virus pseudotyped with the envelope of the ADA HIV1 strain, although all the variants were less able to inhibit entry (Figure 4B).

Further investigations using replication-competent virus entry into CEMx174-CCR5 cells and peripheral blood mononuclear cells (PBMC) were performed. The CEMx174-CCR5 cells were infected with replication competent SF162 virus after pretreatment with chemokine. p24 concentration was determined after 5 days using ELISA (ref. 26). These results were compared to the [p24] released from cells that had not been exposed to any chemokine. The extent of reduction by chemokine treatment determined the percentage of inhibition of HIV entry. Similarly, 5x10⁵ PBMC were infected with replication-competent SF162 virus. PBMC were purified from HIV1 seronegative donors, PHA/IL-2 activated, and infected with virus as previously described²⁶. Five days post-

infection [p24] was determined and percentage inhibition of HIV entry calculated relative to chemokine-untreated control as above.

5 The results are shown in Figures 4C and 4D, respectively (\square = LD78 β ; \diamond = LD78 β -4; \circ = LD78 α ; Δ = LD78 α -4; X = hRANTES; \oplus = AOP-RNATES). These studies suggest that in addition to being by far the most effective LD78 variant in suppressing HIV entry into target cells, LD78 β is also more potent than
10 RANTES, although not quite as effective as the N-terminally modified form of this chemokine, AOP RANTES (ref. 19). Note that RANTES and LD78 α -4 can enhance HIV entry into PBMCs at the lower concentrations tested (Figure 4D). These results present LD78 β as the most potent naturally-occurring
15 inhibitor of HIV1 entry through CCR5. Variants of this protein, such as AOP-linked LD78 β , may exhibit enhanced HIV1 entry inhibition and/or receptor antagonism and have potential as HIV1 therapeutics.

20 LD78 α -4 and LD78 β -4 behave in a similar fashion in all assays we have performed, and we therefore believe that it is the proline residue at position 2 of LD78 β that is crucial for its strong interaction with D6 and for its increased binding to, and signalling through, CCR5. It is likely that proline
25 2 in other β -chemokines is necessary for high affinity binding to D6. This, however, is not sufficient for D6 interaction, as SDF1 also contains a proline residue at position 2 yet shows no potential to bind to D6 (data not shown). Interestingly, this residue in SDF1 is crucial for
30 activation of its receptor CXCR4, which acts as an entry cofactor for T-tropic HIV1 strains²⁰⁻²³, and mutation of this residue to glycine generates a high affinity CXCR4 antagonist²³. Thus, proline 2 must be presented in the context of a β -chemokine to permit high affinity D6

interaction, whilst with CCR5 other domains are likely important in restricting the ligands for this receptor to MIP-1 α , 1 β , RANTES and MCP2. It is intriguing that both major HIV entry coreceptors have a preference for a proline residue at position 2 during receptor activation.

Murine MIP-1 α consistently behaves in a similar fashion to LD78 β , by binding avidly to murine and human CCR5 and D6, and slightly less well to CCR1 (ref. 15 and data not shown) and by being generated consistently in a 'full length' form. LD78 α , on the other hand, either as a full length or -4 variant protein, only binds well to CCR1. We conclude therefore that LD78 β is the functional human homologue of murine MIP-1 α : LD78 α should be considered to be a related, but functionally distinct chemokine. To simplify the nomenclature of these two non-allelic variants and emphasise the importance of proline 2, we propose that the LD78 β gene be renamed MIP-1 α P and that LD78 α be called MIP-1 α S, indicating the change in predicted amino acid sequence to serine at this position.

The LD78 β /MIP-1 α P copy number varies between individuals, and this gene can in fact be absent from some individuals¹³⁻¹⁴. β -chemokine production has been reported to be associated with a number of inflammatory and autoimmune diseases¹, and deletion of the MIP-1 α gene in mice dramatically alters responses to several infectious agents²⁴. LD78 β /MIP-1 α P gene dosage may alter an individuals response in these pathological situations. Moreover, in light of the potent HIV entry inhibition by LD78 β /MIP-1 α P described here, it is of considerable interest to test whether gene copy number affects the rate of progression to AIDS in HIV-infected individuals.

Example 5: Receptor binding studies with AOP-MIP-1 α P.

An AOP derivative of MIP-1 α P was synthesised using solid phase synthesis (by Gryphon Sciences, CA, USA) in which an AOP-proline group was attached to the N-terminal of LD78 β in place of proline.

5

(An alternative is to use similar techniques to those of Simmonds et al (Ref. 19), by generating an LD78 β variant with serine in place of alanine at position 1, and modifying the serine to generate an aldehyde group which is then reacted to the hydroxylamine derivative of aminooxypentane to provide the AOP-LD78 β molecule.)

10

The AOP-LD78 β molecule was tested for binding to the human CCR5 receptor expressed in CHO cells using the methods outlined above in Example 2, but with labelled AOP-MIP-1 α P being used at 20 nM.

15

The displacement of I¹²⁵-mMIP-1 α (\diamond , \square) or AOP-MIP-1 α P (Δ) is shown in Figure 5. As indicated, the results demonstrate that AOP-MIP-1 α P is a more potent blocker of murine MIP-1 α binding to CCR5 than MIP-1 α P itself. The basis for this enhanced blocking activity appears not to be due to an enhanced affinity of AOP-MIP-1 α P for CCR5 as AOP-MIP-1 α P displacement of AOP-MIP-1 α P from this receptor shows it to have a binding affinity that is broadly similar to that seen with the unmodified MIP-1 α P. It is likely that the enhanced blocking seen with AOP-MIP-1 α P is a reflection of the enhanced ability of this ligand to down-regulate cell surface receptor levels thus effectively denuding the cell surface of receptors. Essentially this is the same mechanism that is used by AOP-RANTES in blocking CCR5 function.

20

25

30

The fact that MIP-1 α P is similar in potency to AOP-RANTES in anti HIV assays and that the AOP-MIP-1 α P variant has an approximately 5-10 fold enhanced ability to block CCR5

35

binding of MIP-1 α P indicates that it is likely to be a more potent anti-HIV therapeutic than AOP-RANTES. This means that clinically relevant concentrations of AOP-MIP-1 α P will be more easily achieved than with AOP-RANTES indicating that AOP-MIP-1 α P may be a more attractive clinical agent.

Example 6: Receptor signalling studies with AOP-MIP-1 α P.

The studies of Example 3 were repeated using the AOP-MIP-1 α P. It was found that AOP-MIP-1 α P is similar in potency to its unmodified counterpart in signalling assays on either CCRs 1 or 5. This again supports the conclusion of Example 5 that the major enhancement of blocking potency seen with AOP-MIP-1 α P is a consequence of more efficient receptor down regulation.

Example 7: Receptor down-regulation studies.

CHO cells expressing human CCR5 receptor as described in example 2 were incubated with 100 nM of AOP-MIP-1 α P or MIP-1 α P for 1 hour, then washed and incubated with medium for the specified time periods indicated on Figure 6. An azide containing binding assay (see Refs. 15 & 16) was performed to measure the downregulation of receptors. As indicated, a 1 hour incubation with AOP-MIP-1 α P induces an 80% downregulation of cell surface receptor levels compared with the 50% seen with MIP-1 α P. Even more significantly is the observation that the AOP-MIP-1 α P downregulation is much more prolonged than that seen with the unmodified MIP-1 α P. In fact, over the 60 minute recovery period studied in the experiment, the CCR5 receptor levels in the AOP-MIP-1 α P treated cells barely recovered from their 20% level whilst those in the MIP-1 α P treated cells recovered essentially completely within 40 minutes.

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CLAIMS

1. A method of inhibiting the entry of an HIV-1 virus into a CD4⁺ cell which method comprises:

5 bringing said cell and said virus into contact in the presence of a polypeptide comprising the LD78 β isoform of MIP-1 α (MIP-1 α P) under conditions wherein said polypeptide binds to a CCR5 receptor on the surface of said cell and inhibits entry of said virus into the cell.

10 2. A diagnostic method for determining the likely effectiveness of LD78 β (MIP-1 α P) therapy in a patient infected with, or at risk of infection from, HIV, which method comprises:

15 isolating a sample containing CD4⁺ T-cells from said patient;

bringing said T-cells into contact with a polypeptide comprising the LD78 β isoform; and

20 determining the extent to which said polypeptide can bind to said cells.

3. A method according to claim 2 wherein said sample is a human body sample selected from blood or serum.

25 4. A method according to any one of the preceding claims wherein said LD78 β isoform comprises an amino-oxypentane moiety at its N-terminus.

30 5. The use of a polypeptide comprising the LD78 β isoform of MIP-1 α for the manufacture of a medicament for the treatment of HIV infection.

6. Amino-oxypentane LD78 β (AOP-MIP-1 α P).

7. AOP-MIP-1 α P for use in a method of treatment of the human or animal body.
8. A method of treating HIV infection which method
5 comprises administering to a patient infected with HIV an effective amount of a chemokine selected from the group MIP-1 α P and AOP-MIP-1 α P.
9. A method of diagnosis or prognosis of the course of HIV
10 infection in an individual which method comprises determining the copy number in the genome of the gene for MIP-1 α P or its copy number in a body sample.

FIG. 1

| | | | | | | | | | | | |
|--------------------------------------------|-------|-------------------------|------------|------------|------------|------------|------------|------------|---------|---|---|
| hMIP-1β | ----- | -S ^F YSSDT.T | PCCFAYIARP | LPRAHKEVF | Y.TSGKCSNP | AVVFVTKNR | QVCANPEKKW | VREYINSLEM | S----- | + | + |
| mMIP-1β | ----- | -A ^P MGSDPPT | ACCFSYTARK | LPNFWVDYY | E.TSSLCSOP | AVFQTKRSK | QVCADPSESW | VQEYVYDLEL | N----- | + | + |
| hMIP-1α { hLD78α/MIP-1αS hLD78β/MIP-1αP | ----- | -A ^P MGSDPPT | SCCFSYTSRQ | LHRSFVMDYY | E.TSSLCSKP | AVVELTKRGR | QICNPSPQW | VTEYHSELEL | N----- | + | + |
| | ----- | -A ^S LADTPT | ACCFSYTSRQ | IPQNFYADYF | E.TSSQCSKP | GVIFLTKRSP | QVCADPSESW | VQKYVSDLEL | SA----- | - | - |
| | ----- | -A ^P LADTPT | ACCFSYTSRQ | IPQNFYADYF | E.TSSQCSKP | SVIFLTKRGR | QVCADPSESW | VQKYVSDLEL | SA----- | ? | ? |
| | ----- | -A ^P YGADTPT | ACCFSY.SRK | IPRQFVVDYF | E.TSSLCSOP | GVIFLTKRNR | QICADSKETH | VQEYITDLEL | NA----- | + | + |

FIG. 2

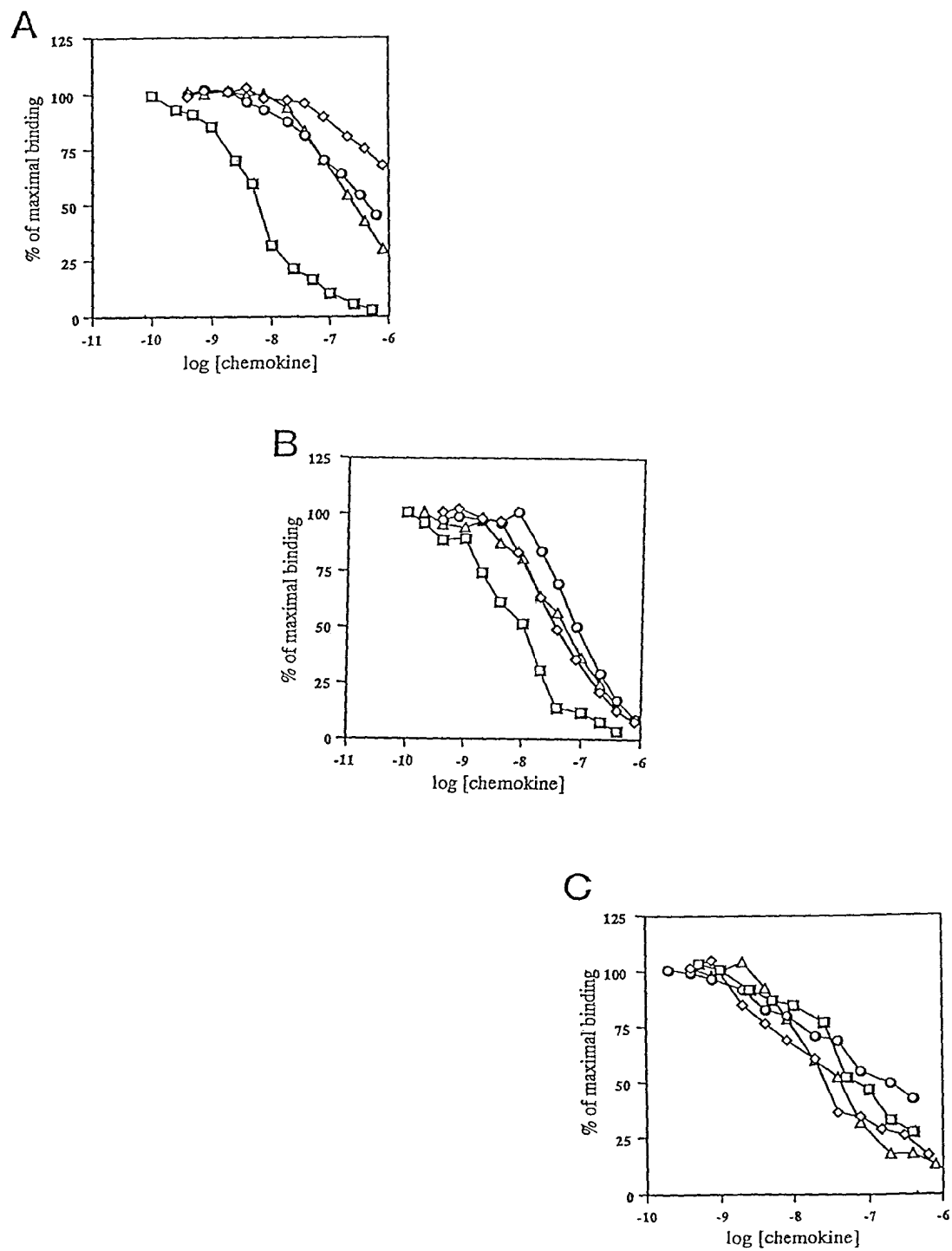


FIG. 3

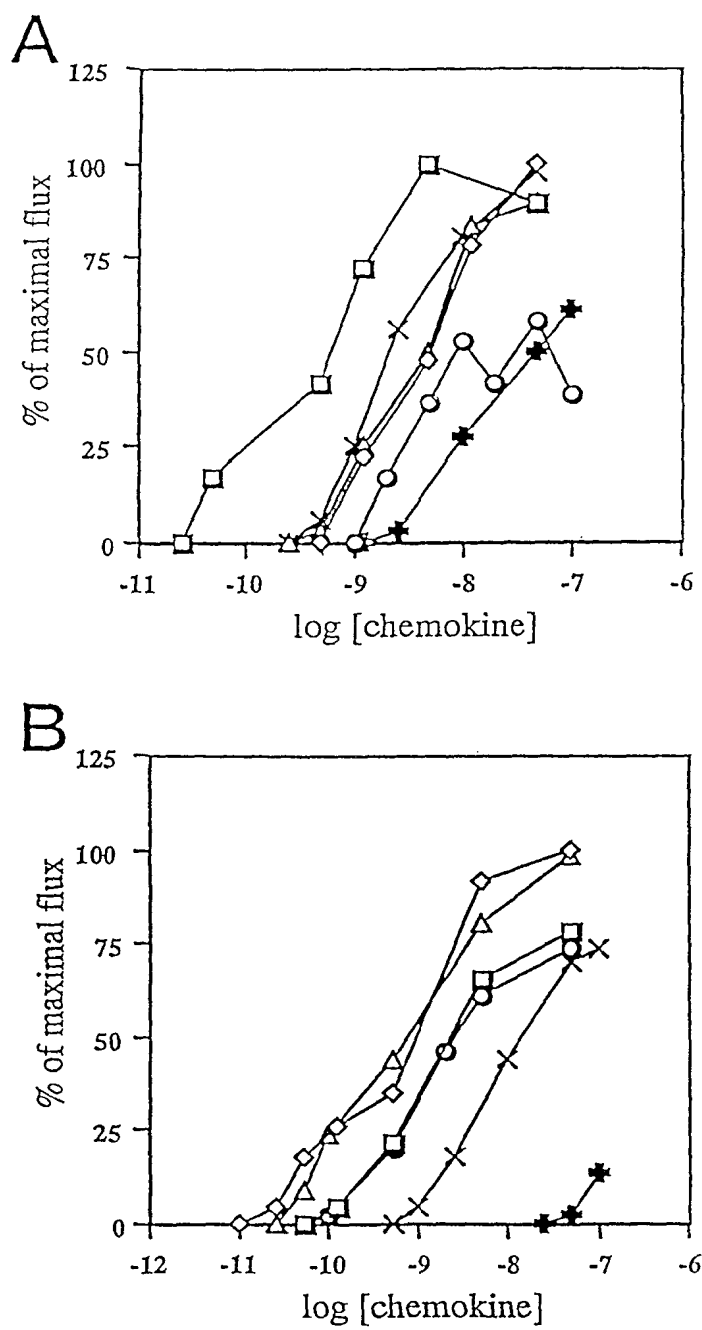


FIG. 4

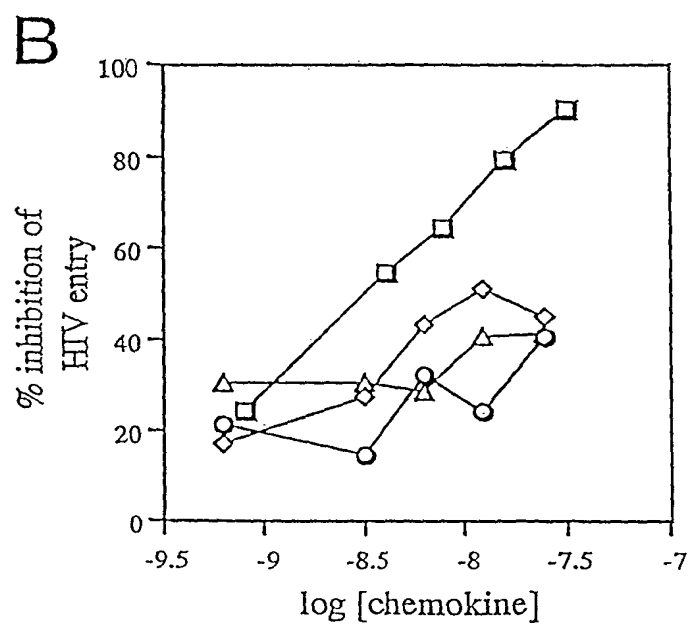
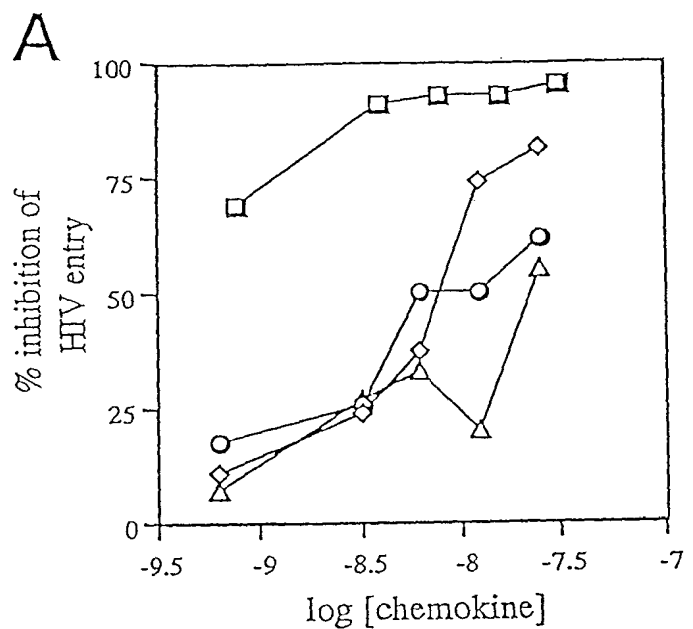
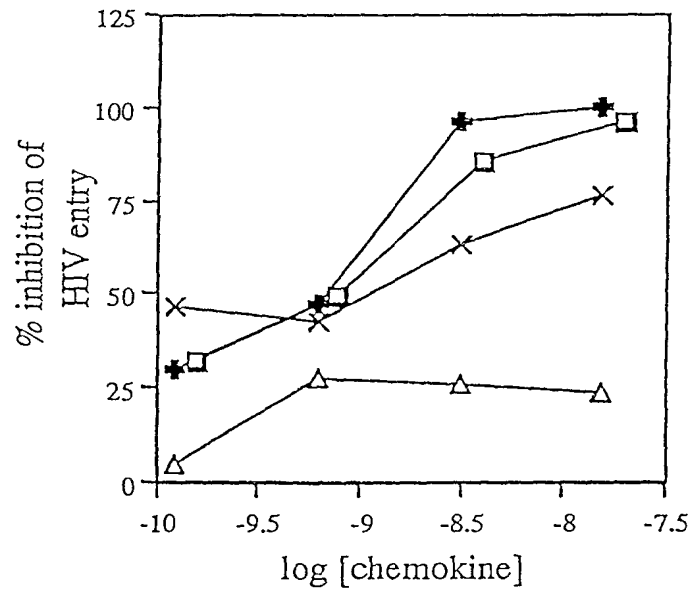
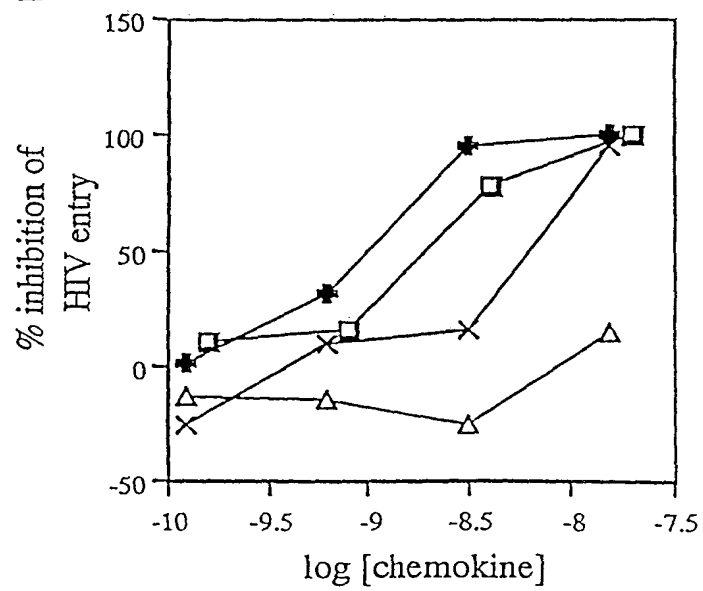


FIG. 4

C



D



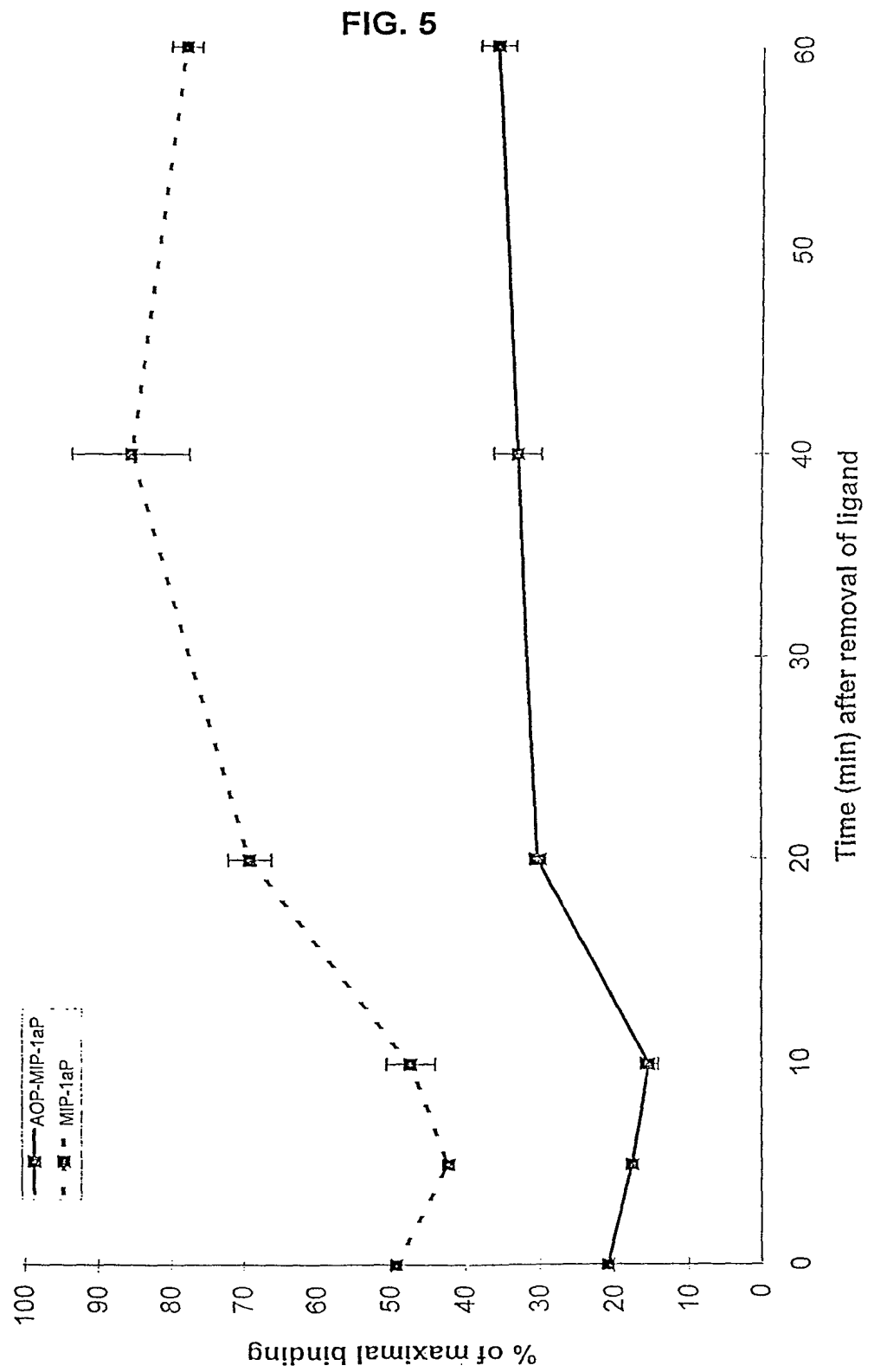
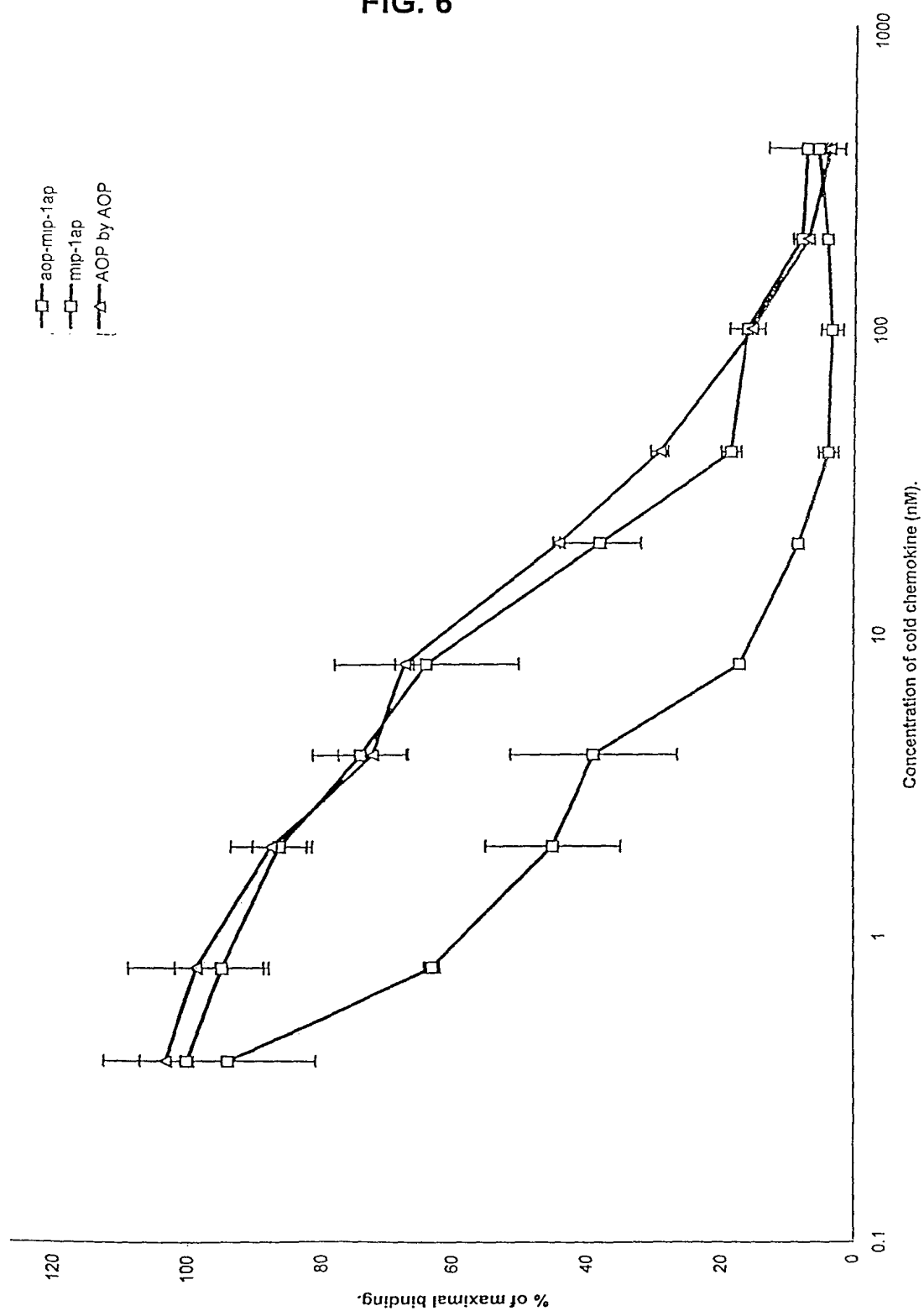


FIG. 6



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03059

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/52 A61K38/19 G01N33/68 A61K47/48 C07K1/107
C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A | SIMMONS G ET AL: "POTENT INHIBITION OF HIV-1 INFECTIVITY IN MACROPHAGES AND LYMPHOCYTES BY A NOVEL CCR5 ANTAGONIST" SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 276, 11 April 1997 (1997-04-11), pages 276-279, XP002914261 ISSN: 0036-8075 cited in the application the whole document --- -/- | 1-8 |



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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4 February 2000

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23/02/2000

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Le Cornec, N

INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A | <p>COCCHI F ET AL: "IDENTIFICATION OF RANTES, MIP-1ALPHA, AND MIP-1BETA AS THE MAJOR HIV-SUPPRESSIVE FACTORS PRODUCED BY CD8+ T CELLS"</p> <p>SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 270, 15 December 1995 (1995-12-15), pages 1811-1815, XP000616644 ISSN: 0036-8075 cited in the application the whole document</p> | 1-8 |
| A | <p>NAKAO M ET AL: "STRUCTURES OF HUMAN GENES CODING FOR CYTOKINE LD78 AND THEIR EXPRESSION"</p> <p>MOLECULAR AND CELLULAR BIOLOGY,US,AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, vol. 10, no. 7, 1 July 1990 (1990-07-01), pages 3646-3658, XP000606883 ISSN: 0270-7306 cited in the application page 3649 -page 3651; figures 2,3 page 3655, left-hand column</p> | 9 |
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INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/GB 99/03059

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| P,X | <p>R.J.B. NIBBS ET AL: "LD78beta, anon-allelic variant of human MIP-1alpha (LD78alpha), has enhanced receptor interactions and potent HIV suppressive activity"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 274, no. 25, 18 June 1999 (1999-06-18), pages 17478-17483, XP002129767</p> <p>AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD., US</p> <p>ISSN: 0021-9258</p> <p>the whole document</p> | 1-9 |
| P,X | <p>P. MENTEN ET AL: "The LD78beta isoform of MIP-1alpha is the most potent CCR5 agonist and HIV-1-inhibiting chemokine"</p> <p>JOURNAL OF CLINICAL INVESTIGATION, vol. 104, no. 4, August 1999 (1999-08), pages R1-R5, XP000867805</p> <p>the whole document</p> | 1-8 |
| P,X | <p>X. XIN ET AL: "Enhanced anti-HIV-1 activity of CC-chemokine LD78beta, a non-allelic variant of human MIP-1alpha/LD78alpha"</p> <p>FEBS LETTERS., vol. 457, no. 2, 27 August 1999 (1999-08-27), pages 219-222, XP002129769</p> <p>AMSTERDAM NL</p> <p>the whole document</p> | 1-9 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 99/ 03059

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claim 1,4 (as far as they concerns an in vivo method) and claim 8 are directed to a method of treatment of the human/animal body (rule 39.1 IV PCT), the search has been carried out and based on the alleged effects of the compound/composition.

And Although claim 9 (as far as it concerns an in vivo method) is directed to a diagnostic method practised on the human/animal body (rule 39.1 IV PCT), the search has been carried out and based on the alleged effects of the compound/composition.